(DEVELOPMENTAL)

LABORATORY METHODS

IN

CLINICAL BACTERIOLOGY

Course No. 8380-C

TECHNIQUES

BACTERIOLOGY TRAINING BRANCH

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BLOOD CULTURES

Introduction.

Bacteria may be found sporadically or persistently in the blood of a patient. The bacteremia may be due to aerobic or anaerobic organisms and may be prolific or very fastidious. Covering such a broad spectrum of bacteria with the least complicated substrate and the easiest and most practical collection procedure is a challenge to the system which the bacteriologist has available.

The problem of clotting of the specimen in the medium has been reduced substantially with the use of sodium polyanethol sulfonate (SPS). This substance appears to increase the yield of organisms isolated from blood by preventing clotting and inactivating leucocytes, complement, and certain aminoglycoside and polypeptide antibiotics.[1,2,3] It inhibits a species of anaerobic cocci[4] and there is a report indicating a sensitivity of N. meningitidis to SPS.[5] SPS is superior to the use of oxalate, citrate and ethylenediaminetetracetic acid because of their inhibitory action. A large volume of specimen, usually 10 ml of blood, increases the possibility of isolating the bacterial agent. The blood should be diluted at least one part blood in nine parts broth to insure the best yield.

Atmosphere and selection of medium is very important for certain organisms. Pseudomonas is isolated more often if the atmosphere contains adequate oxygen.[6] Thioglycollate broth has been widely used as an all purpose broth but it is becoming apparent that it is not the best selection for organisms such as Streptococcus pneumoniae, Haemophilus influenzae, Pseudomonas aeruginosa and others, both aerobic and anaerobic.

Most commercial bottles come under vacuum with an atmosphere of 5-10% CO_2. They may be vented or not, according to their use for aerobes or anaerobes. There are a variety of commercial media and several companies will prepare media using the directions of the particular laboratory. The following procedure is presented as a method which will yield most agents of bacteremia. It will not allow the growth of Leptospira and probably not Brucella or Francisella. For these, special procedures and media should be followed. An excellent review of blood culture procedures is presented in the first paper of the Cumitech series published by the American Society for Microbiology.[7]

Blood culture media.

The method described uses commercially available blood culture bottles under vacuum. Due to the vacuum, the bottles are best used with a commercial blood collecting unit rather than a syringe and hypodermic needle for venipuncture. For collecting from an infant, a needle and syringe may be necessary.

One aerobic and one anaerobic blood culture bottle are used with each blood specimen. Each bottle has 50 ml of broth media with 0.025% sodium polyanethol sulfonate as an anticoagulent and 10% CO_2 in the atmosphere. Each bottle receives 5 ml of patient's blood to give a dilution of 1 ml of blood to 9 ml of broth. Penicillinase may be added to either bottle immediately upon collection of the specimen.

The aerobic blood culture bottle should contain a broth medium which will support the growth of essentially all aerobic and facultative bacteria. Suitable media include tryptic or trypticase soy broth, Columbia broth, brain heart infusion broth, and Brucella broth. Filtered air is allowed to enter this bottle and it is subcultured routinely on the first, second, and fifth day of incubation.

Suitable media for the anaerobic blood culture bottle include Columbia broth with 0.05% cysteine, Schaedler broth, pre-reduced brain heart infusion broth, and tryptic or trypticase soy broth. This bottle is not vented and is subcultured routinely on the second and fifth day of incubation.

Collection of specimen.

1. Preparatory steps.

    a. Label both bottles with patient's identification. Label the bottle to be vented "aerobic" and the other bottle "anaerobic."

    b. Wash the site for venipuncture with a swab saturated in 70% alcohol and swab the surface with an applicator of 2% tincture of iodine. Allow iodine to dry and remove the residue with another alcohol sponge. The vein should not be touched after cleaning.

2. Venipuncture and inoculation of bottles.

    a. Remove cotton-filtered shield (save) from needle and perform venipuncture. Unclamp hemostat and allow blood to flow into needle. Reclamp the hemostat and uncover the anaerobic bottle. Insert the free needle into this bottle, unclamp the tubing allowing the blood to reach the mark that indicates the addition of 5 ml. Reclamp.

    b. Remove needle from anaerobic bottle and insert into aerobic bottle. Allow to flow to mark. Clamp.

    c. Remove needle from patient's arm and cover needle with the sterile cotton-tipped filter that was saved when first removed. Unclamp hemostat and allow air to flow through filter into bottle until vacuum is filled. Remove needle, wash both bottle tops with alcohol and replace caps for cleanliness.

    d. Send to laboratory immediately.

3. Frequency and number of specimens.

   a. Collect three sets of specimens.

   b. Three specimens should be taken in 24 hours, preferably at one hour intervals.

   c. Special situations.

      (1) On patients admitted with life-threatening symptoms, collect two sets of blood cultures immediately.

      (2) On patients on therapy with poor response, collect a maximum of six sets of blood cultures, each taken immediately before antibiotic therapy is given.

4. Problems encountered collecting specimen.

   a. If needle is inserted into the bottle before tubing is clamped, the vacuum will be lost.

   b. The use of a syringe or unfiltered needle for ventilation with vacuum bottles can be a source of contamination if surface air is pulled into the bottle.

   c. The commercial collecting unit is more expensive than a disposable syringe and needle; also, it requires more education of personnel to the technique than does the syringe method.

   d. The blood must flow to the end of the tubing before inserting the needle into the anaerobic bottle or air will be introduced into the system.

   e. The vacuum will create a problem when adding blood to the bottle from a needle and syringe. Care must be taken, not to introduce unsterile air into the bottle. Also, there is a chance of allowing the entire 10 ml to be pulled into one bottle.

   f. The aerobe bottle must be entered last in order that it be ventilated without reentry. There is a possibility that high concentrations of CO_2 can slow or inhibit growth.

Initial handling of blood culture bottles in the laboratory.

1. Check bottles for proper labeling.

2. Identify with laboratory number.

3. Separate into aerobic and anaerobic sets.

4. Incubate both bottles at 35°C.

Schedule for examination of cultures.

1. Observe all bottles macroscopically daily for seven days. Gram stain and subculture any obviously positive bottles. Report any positive results to physician.

2. Gram stain and do blind subcultures on all aerobic bottles at 18-24 hours.

3. Gram stain and do blind subcultures on all aerobic and anaerobic bottles at 48 hours. Send out tentative reports on all cultures.

4. Do blind subcultures on all aerobic and anaerobic bottles at 5 days.

5. Do Gram stain and discard all bottles and subcultures at 7 days. Send out final reports.

Details for examination and subculturing of blood culture bottles.

1. Remove bottles from incubator without disturbing sedimented blood layers and examine under a bright light for evidence of turbidity, hemolysis, gas production, or bacterial colonies in or on the blood layer.[7]

2. Swab the tops of bottles with iodine and alcohol and burn the remaining alcohol from the top before entering the bottle with a needle and syringe.

3. If growth is seen, remove at least 1 ml of blood broth and inoculate a chocolate agar plate, two blood agar plates, and a trypticase broth for sensitivity testing. Incubate the chocolate plate and one BAP in CO_2 and one BAP in an anaerobic atmosphere, all at 35°C.

   a. Spread a drop of remaining culture fluid on a slide and Gram stain immediately. Examine and call physician without delay to report positives. In addition, make a notation for the infectious disease staff.

   b. Based on the bacterial morphology, do appropriate biochemicals from the blood culture broth to identify the isolate.

   c. Perform sensitivity tests from the TSB after about 4 hours incubation or from the original blood culture broth.

4. For blind subcultures from the aerobic bottles, place the inoculum on 1/4 of a chocolate agar plate and incubate the plate at 35°C in CO_2. Examine in 24 hours. Reincubate negative plates up to three days.

5. For blind subcultures from the anaerobic bottles, place the inoculum on 1/4 of a prereduced blood agar plate and incubate at 35°C in a Gaspak jar or anaerobic chamber for 48 hours before opening.

Special procedures and media.

1. Pour-plate method.

   a. Melt 20 ml of blood agar base and cool to 45°C.

   b. Add 1 ml of patient's blood to each of two sterile Petri dishes.

   c. Pour agar and rotate until blood is mixed with agar.

   d. Allow to solidify and incubate one plate anaerobically and one in CO_2, both at 35°C.

2. Examination of pour-plates.

   a. Examine plates at 24 hours from the CO_2 atmosphere. Look for subsurface colonies.

   b. Count colonies and record number of organisms per ml.

   c. Gram stain, subculture and identify isolates.

   d. Reincubate negative plates for three days before discarding as negative.

3. Membrane filter for yeast. See Mycology Manual.[8]

4. Hypertonic culture medium for cell wall-deficient bacteria.

   Even though the role of cell wall-deficient bacteria is not established, there is some evidence that cells damaged by antimicrobial agents may be induced to grow in a hypertonic medium. There are conflicting reports for the use of this medium for blood cultures. A hypertonic medium can be made by adding 10% sucrose to one of the acceptable blood culture broths.

SPINAL FLUID

Introduction.

It is advantageous for the cerebro-spinal fluid specimen to arrive in the laboratory in a sterile, flat-bottomed, screw-cap centrifuge tube. These tubes are commercially available and keep contamination to a minimum for both carrier and specimen.

Spinal fluids should always get emergency handling and should never wait for other cultures to be processed. The carrier should place the specimen in the hands of the person in charge of processing the culture and not in the refrigerator or on the bench.

Procedure.

1. In presence of carrier, check specimen for identification.

2. Label specimen.

3. If specimen is cloudy with white cells, remove a drop with a sterile pipette and spread on clean slide to dry.

4. If it is not cloudy, centrifuge 10 minutes at 1500 rpm.

5. With a pipette, plant either the cloudy fluid, or the sediment after centrifuging, to a chocolate agar plate, 2 sheep blood plates and a TSB. Add one drop to each of the plates and 1 ml to the broth. Add a drop to a clean slide but do not spread as is done on a cloudy specimen for staining.

6. Incubate the chocolate and one BAP in a candle jar at 35°C.

7. Incubate one BAP anaerobically.

8. The slide should be dry at this time. Gram stain and read immediately. Do a methylene blue stain if there is doubt about morphology.

9. Report positives by telephone to physician.

10. If negative do an India ink and acid fast stain if there were cells present in the CSF.

24 hour reading.

1. Examine plates and return negatives to incubator.

2. Gram stain positive cultures and report findings to a physician by telephone.

3. Gram stain broth and sub-culture appropriately if there is evidence of growth.

Report plates as negative after 48 hours incubation.

Description of organisms most frequently isolated from CSF.

1. Haemophilus influenzae: 6 months through childhood.

   Morphology: tiny pleomorphic gram negative rods, usually smaller and more fragmentary or less rod-like than enteric rods which are found in CSF. Least discernible of any orgamisms found in CSF especially if few in number. This organism grows well on chocolate agar containing heme and yeast extract or a synthetic NAD, and does not grow on sheep blood agar without these additives. This distinguishes it from other organisms found in CSF. Typing sera for group b is available and satisfactory. This is the type most often found in CSF. Quellung test is often unsatisfactory for identification. Can be oxidase positive; therefore, a Gram stain must be done. Ampicillin resistant H. influenzae have been found.

2. Streptococcus pneumoniae: Adults and children, primarily in older adults.

   Morphology: Gram positive cocci in pairs. The free ends of the diplococci are pointed instead of spherical in most cases and referred to as lancet shaped. They can occur in chains and be coccoid but usually not until grown in broth. Capsule is distinct enough to give positive Quellung with omniserum and with individual groups. Flourescent conjugate available and satisfactory for spinal fluids. Grows on blood or chocolate plates producing green colonies that are often mucoid. Can be identified as pneumococcus by bile solubility or optochin disc. Sensitivity testing not required.

3. Neisseria meningitidis: School age children through adulthood, greatest in young adults.

   Morphology: Gram negative diplococci, easily distinguished if adequately stained and decolorized. Adjacent sides of the cocci are slightly flattened. Not pleomorphic and growths on both blood and chocolate agar as a round, smooth, transparent and butyrous colony becoming rubbery with age. These are oxidase positive.

Grouping serum for A, B, C, D is available. Non-groupables by this method should be sent to reference laboratories for additional grouping. Fermentation of glucose and maltose is indicative of N. meningitidis. ONPG for lactose should be included in the carbohydrates tested.

4. Listeria monocytogenes: New born to 2 months of age or the compromised patient, any age.

Morphology: Tiny, fragmentary, gram positive rods. Easily confused with diptheroids or streptococci.
F-A conjugate is available.
Should be submitted to reference laboratory for typing.
Grows on sheep blood plate with beta hemolysis under the colony.
Grows on chocolate agar. Colony is small and white somewhat resembling a Group B Streptococcus. Typical tumbling motility can be detected in young broth cultures incubated at 25°C.
Grows on and blackens bile esculin and is catalase positive.
Disc diffusion sensitivity is reproducible, although interpretation of zone size has not been standardized.

5. Escherichia coli: Adults, often with urinary tract infections.

Morphology: More rod shaped and larger than other gram negative rods mentioned.
Colony is moist, shiny and larger than any mentioned previously.
Sensitivity should be started immediately when the above rods are detected. A preliminary sensitivity can be done directly from CSF but should be confirmed by a standard Kirby-Bauer after isolation of the organisms.

6. Klebsiella pneumoniae: Adults, usually with pneumonia.

Morphology: Gram negative rods with bi-polar staining. A capsule is usually observed when stained with methelyene blue.
Colony is moist, usually mucoid and as large as E. coli.
Sensitivity done immediately as described for E. coli.

7. Streptococcus:

Group B: Newborns
Group A: Patients from surgery, hydrocephalics, etc.
Group D: Adults with urinary tract infections and other foci.

Morphology: Gram positive cocci, occurring singularly, in pairs or chains. Not lancet shaped although some bacteria have bizarre shapes. Beta hemolytic, colony may vary greatly according to group or intra group. Colony morphology not reliable.
Since the organism may be a Group D, non-bovis streptococcus, sensitivity should be started immediately.

8. Staphylococcus aureus: Any age, trauma, abscess, other foci. S. epidermidis in compromised patient.

Morphology: Gram positive cocci, occurring singularly, in pairs, and clusters. Colonies may be buff white, or golden yellow. Coagulase test is done from the colony.
Catalase positive.
Sensitivity done immediately.

9. Crytococcus neoformans: Older compromised patient.

Morphology: Large capsule around budding yeast as seen with the aid of an India ink preparation. Colonies white to buff color, mucoid. If yeast are seen in India ink preparation, a brain heart infusion agar plate should be inoculated for isolation of crytococci. Occasionally a rare strain of cryptococcus will have two colony types. The rough will have rudimentary mycelia. The smooth will have only budding yeast. This culture should not be considered contaminated. If possible, filter membrane culturing should be done on CSF suspected of cryptococcal infection.

10. Mycobacterium tuberculosis:

Morphology: Acid fast bacilli, red with carbol fuchsin; flourescent with auramine stain. Milipore filter membrane culturing recommended.

URINE COLLECTION AND CULTURE

. Introduction.

"Care in collection, storage and transport of urine specimens for bacteriological examination is of the utmost importance. The best laboratory techniques for counting and identifying bacteria are of little value if the specimen is not collected properly and delivered to the laboratory without delay or under appropriate refrigeration. Contaminating bacteria from the perineum can multiply rapidly at room temperature and can invalidate the counts done either microscopically or by culture."[9]

Because contamination is so common, colony counting is used to distinguish simple contamination of the urine due to the collection method from actual infection of the urinary tract. A true infection is more likely if there is a greater number of bacteria present. The term "bacteriuria" is generally taken to mean there are 100,000 or more bacteria per ml of urine; even though, it is not excluded that a patient may have a significant infection with fewer bacteria per ml.[9,10,11]

Counts between 10,000 and 100,000 bacteria per ml of urine are of doubtful significance and another specimen should be submitted to the laboratory. These counts usually change on the second specimen and better reflect infection or contamination.

. Types of collection.

1. Catherization - recommended when either surgically required or indwelling.

2. Clean-void midstream - quite adequate if patient is clean and if specimen is sent directly to the laboratory or refrigerated in the interim.[12]

3. Suprapubic - most likely to avoid contamination - recommended if absolute verification of culture is needed.[13]

. Methods of collecting specimens.[10]

1. Tapping catheter tube.

   a. Wash tubing well with 70% alcohol sponge.

   b. Puncture tube with needle and syringe.

   c. Withdraw about 20 ml of urine from patient.

   d. Send to laboratory immediately.

Catheter should not be cultured upon removal because the catheter is contaminated as it is removed from urethra.[14]

NOTE: Silicone catheter will leak if punctured.

2. Clean-void midstream.

The orifice must be thoroughly cleaned and the following supplies are needed:

Five or more sterile gauze squares.
Tincture of green soap.
Warm water.
Wide mouth receptable for collection.
Set of graphic instructions which may or may not be reinforced orally.

a. Patient removes all undergarments and washes hands thoroughly.

b. Female: "Spreads herself" holding both sides of labia back with one hand.

Male: Retracts foreskin before washing head of penis.

c. Clean exposed area with one sponge wet with green soap passed from front to back. Discard sponge. Wash three more times with soap in the same manner, discarding sponges with each backward stroke. Use one wet sponge to remove soap.

d. The patient voids and catches urine in container.

e. Fill container half full and give to attendant without holding top of container.

f. Specimen is capped by attendant and the time of collection written on the label.

3. Suprapubic puncture - collected by physician.

Supplies needed:

3% iodine and 70% alcohol.
1 1/2 inch 19-20 gauge needle.
20 ml syringe.
Container for urine or rubber stopper for needle if specimen is kept in syringe.

a. Check and schedule patient with full bladder before procedure is attempted.

b. Paint skin of patient with iodine and allow to dry. Area covered is from point in midline about 1/3 the distance from symphysis pubis to the umbilicus.

c. Pass needle through skin into bladder maintaining negative pressure on syringe after inserting through skin.

d. Place urine in container or insert needle into sterile rubber stopper and record time of collection.

e. Take directly to the laboratory.

Receipt of specimen in the laboratory.

1. Check for diagnosis.

2. Check for time of collection. If time of collection is not available, specimen should be refrigerated and held until time is verified or another specimen is collected. Specimen is acceptable up to 24 hours if it has been refrigerated.

Bacterial counts from urine specimens.

The urine must be counted for the number of bacteria per ml. This is done by culture but a rapid tentative report may be obtained by doing a Gram stain on the undiluted specimen. Counts from culture can be obtained using a calibrated loop, pipette dilution, or a pour plate method. The pour plate procedure is the reference method and can be used to control the accuracy of either the calibrated loop or pipette dilution method.

1. Microscopic examination.

   a. Place one 4 mm loopful of urine on a slide and allow to dry without spreading.

   b. Fix by adding a drop of 95% alcohol and allow to dry again.

   c. Gram stain and read with oil immersion objective. The presence of one or more bacteria per field is indicative of a count greater than 100,000 per ml.

   d. Leucocytes in a specimen, with or without bacteria, should be reported.

2. Calibrated loop method.

   a. Shake specimen well to insure even distribution of any organisms present.

b. Dip a sterile 0.01 ml calibrated loop directly into and out of the urine and streak down the center of a blood agar plate.

c. Flame and cool the loop; redip, and streak an EMB plate.

d. Dip a sterile 0.001 ml loop into urine and streak down the center of another blood agar plate.

e. Spread the inoculum evenly on all plates using sterile glass spreaders or a loop and incubate the plates for 18-24 hours at 35°C.

3. Pipette dilution procedure.

a. Shake specimen well to insure an even distribution of organisms.

b. Prepare a 1:10 dilution of urine by adding 1 ml of urine to a 9 ml water blank. Prepare a 1:100 dilution by adding 1 ml of the 1:10 dilution to a 9 ml water blank. Mix the dilutions thoroughly.

c. Measuring between the lines of a serological pipette (0.2 ml or 1 ml), pipette 0.1 ml of the 1:10 dilution to the center of a blood agar and 0.1 ml to an EMB plate.

d. Pipette 0.1 ml of the 1:100 dilution to the center of a blood agar plate.

e. Spread the inoculum evenly over the plates with a loop or sterile glass spreader and incubate the plates for 18-24 hours at 35°C.

4. Pour plate procedure.

a. Shake specimen well to insure an even distribution of organisms.

b. Prepare a 1:100 dilution of urine as in the pipette dilution procedure or by adding 0.1 ml of urine to a 9.9 ml water blank. Prepare a 1:1000 dilution by pipetting 1 ml of the 1:100 dilution to a 9.0 ml water blank.

c. Add 1.0 ml of the 1:100 dilution to 20 ml of melted blood agar base cooled to approximately 45°C. Mix well.

d. Flame lip of tube containing agar-specimen mixture and pour into sterile Petri dish.

e. Wipe lip of tube with disinfectant-soaked cotton.

f. Repeat steps c, d, and e with the 1:1000 dilution of specimen.

g. Allow agar to solidify and incubate plates overnight.

5. Determination of colony count.

a. The use of two dilutions allows the determination of both high and low counts. Total counts are determined on non-inhibitory media such as blood agar plates. The EMB plate is used to estimate the number of enteric gram negative rods and as a basis for identification.

b. Count colonies on all non-inhibitory plates containing 30-300 colonies and calculate the number of organisms per ml of undiluted urine. If both dilutions contain colonies in the countable range, use an average of the two results for total counts.

c. To determine total counts per ml of undiluted urine, multiply the count obtained by the dilution. In each of the methods listed, the plate count of the lowest dilution is multiplied by 100 and the highest dilution by 1000.

Isolation and interpretation of bacterial counts.

1. Identify organisms on counts under 10,000 only when patient is currently on therapy, or when specimen is taken by bladder puncture.

2. Identify the organisms from 10,000 - 100,000 counts. Hold culture for sensitivity if special request is made by physician.

3. Do antibiotic sensitivity and identification on counts greater than 100,000/ml.

4. Designate the predominate organism in a mixed infection of greater than 100,000/ml.

5. Since mixed infections can occur, two gram negative rods in a specimen would be identified but three gram negative rods would probably indicate contamination. Under these circumstances, do not perform a sensitivity test but request another specimen.

DISC SUSCEPTIBILITY TESTING PROCEDURE

Preparation of medium.

1. Autoclave Mueller-Hinton agar at 121°C for 15 minutes.

2. Cool to 48-50°C.

3. Pour into 15 x 150 mm sterile Petri dishes (glass or plastic) to a depth of 4 to 5 mm (60-80ml). To test certain fastidious organisms such as streptococci, add 5% defibrinated sheep or rabbit blood before pouring the plates.

4. Let the medium harden and allow to stand at room temperature long enough for excess moisture to evaporate. Plates may be incubated up to 30 minutes at 35°C if necessary. There should be no moisture droplets on the surface of the medium or on the Petri dish covers. The final pH of each lot of solidified Mueller-Hinton agar should be 7.2 - 7.4. Adjust the pH with 1 N NaOH or HCL before autoclaving.

5. Sensitivity test plates may be used immediately or may be stored under refrigeration for 1 week or longer if properly sealed to prevent evaporation. Plates must not be allowed to dry out.

Preparation of inoculum.

1. Select 4 to 5 similarly appearing colonies of the organism to be tested with a wire loop from a pure culture or the primary isolation plate.

2. Transfer these colonies (by touching top of each colony) to a tube of 3 to 5 ml Mueller-Hinton or trypticase soy broth.

3. Incubate the tube at 35°C long enough (2 to 8 hours) to produce an organism suspension with moderate cloudiness. Dilute the broth culture with sterile saline or broth to obtain a turbidity equivalent to that of a turbidity standard obtained by adding 0.5 ml of 1.175% barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$) solution to 99.5 ml of 0.36 N (1%) sulfuric acid. The turbidity standard can be stored in the dark at room temperature for up to 6 months provided the tube is sealed to prevent evaporation. The standard must be thoroughly mixed just before use, preferably on a vortex mixer.

Streaking plates.

1. Dip a sterile cotton swab into the inoculum which should be barely turbid.

2. Rotate swab around inside of tube to remove as much excess inoculum as possible.

3. Streak the swab over the agar survace evenly in 3 directions.

4. Allow inoculum to dry for 3-5 minutes with plate closed.

5. Place discs on the agar with a dispenser or sterile forceps. Press discs down gently on the agar with sterile forceps to insure even contact.

6. Place about 9 discs in the outer ring and 2 or 3 discs in the center. Space discs evenly.

   Place antibiotics which diffuse well in the outer circle, and discs which produce smaller inhibition zones (such as vancomycin, colistin, and polymyxin B) in the central area of the plate.

7. Incubate plates immediately, or within 30 minutes at 35°C. Do not use higher temperatures because some methicillin resistant staphylococci may be missed. Do not incubate in a candle jar.

8. Read plates after 18-24 hours incubation.

Reading plates.

1. Read the zone size around each disc under reflected light. Measure zone diameters (including the 6 mm disc) with a ruler on the under-surface of the Petri dish without removing the cover. Carefully prepared templates may also be used to read zone diameters. (A reading of 6 mm indicates no zone.) If blood agar is used, measure the zones from the surface with the cover removed from the plate.

2. End point: complete inhibition of growth as judged by the naked eye except for sulfonamides and Proteus species.

3. Sulfonamides: slight growth (80% or more inhibition) may occur throughout the zone of inhibition because some organisms grow through several generations before sulfonamide takes effect.

4. Strains of Proteus mirabilis and Proteus vulgaris may swarm into areas of inhibited growth around certain antimicrobics. The zones of inhibition are usually clearly outlined and this veil of swarming growth is ignored.

5. If rapid results are desired, zone diameters are often readable after 6-8 hours incubation but should be confirmed after overnight incubation.

Interpret zone sizes as shown in the table which follows:

Table I

ZONE SIZE INTERPRETATIVE CHART

Antibiotic or Chemotherapeutic agent	Disc potency	Diameter of zone of inhibition (mm) Resistant	Intermediate	Susceptible
Penicillin when testing:				
Gram negative enteric organisms and enterococci	10 mcg	11 or less	12 - 13	14 or more
Staphylococci and penicillin G susceptible organisms	10 mcg	20 or less	21 - 28	29 or more
Hemophilus species	10 mcg	19 or less		20 or more
Bacitracin	10 U	8 or less	9 - 12	13 or more
Carbenicillin when testing:				
Proteus sp. and E. coli	100 mcg	17 or less	18 - 22	23 or more
Pseudomonas aeruginosa	100 mcg	13 or less	14 - 16	17 or more
Cephalothin[1]	30 mcg	14 or less	15 - 17	18 or more
Chloramphenicol	30 mcg	12 or less	13 - 17	18 or more
Clindamycin[2]	2 mcg	14 or less	15 - 16	17 or more
Colistin[3]	10 mcg	8 or less	9 - 10	11 or more
Erythromycin	15 mcg	13 or less	14 - 17	18 or more
Gentamicin	10 mcg	12 or less	13 - 14	15 or more
Kanamycin	30 mcg	13 or less	14 - 17	18 or more
Methicillin[4]	5 mcg	9 or less	10 - 13	14 or more
Nalidixic acid[5]	30 mcg	13 or less	14 - 18	19 or more
Neomycin	30 mcg	12 or less	13 - 16	17 or more
Nitrofurantoin[5]	300 mcg	14 or less	15 - 16	17 or more
Penicillin G[6] when testing:				
Staphylococci	10 U	20 or less	21 - 28	29 or more
Other oranisms	10 U	11 or less	12 - 21*	22 or more
Polymyxin B[3]	300 U	8 or less	9 - 11	12 or more
Streptomycin	10 mcg	11 or less	12 - 14	15 or more
Sulfonamides[5,7]	300 mcg	12 or less	13 - 16	17 or more
Tetracycline[8]	30 mcg	14 or less	15 - 18	19 or more
Trimethoprim - Sulfamethoxazole	1.25 mcg 23.75 mcg	10 or less	11 - 15	16 or more
Vancomycin	10 mcg	11 or less	12 - 13	14 or more
Viomycin	30 mcg	9 or less	10 - 11	12 or more

*This category includes some organisms such as enterocci and gram negative bacilli which may cause systemic infections treatable by high doses of penicillin G. Such organisms should be reported susceptible to penicillin G but not to phenoxymethyl penicillin or phenethicillin.

1. The cephalothin disc is used for testing susceptibility to all cephalosporin type antibiotics. This includes cephaloridine, cephalexin, cephazolin and cephapirin. Staphylococci exhibiting resistance to methicillin discs should be reported as resistant to cephalosporin type antibiotics regardless of zone size.

2. The clindamycin disc is used for testing susceptibility to both clindamycin and lincomycin.

3. Colistin and polymixin B diffuse poorly in agar and the accuracy of the diffusion method is thus less than with other antibiotics. Resistance is always significant, but when treatment of systemic infections due to susceptible strains is considered, it is wise to confirm the results of a diffusion test with a dilution method.

4. Of the penicillinase-resistant penicillins, only methicillin is tested since results also apply to cloxacillin, dicloxacillin, oxacillin, and nafcillin.

5. Susceptibility data for nalidixic acid, nitrofurantoin, and sulfonamides apply only to organisms isolated from urinary tract infections.

6. Penicillin G is used to test the susceptibility of all penicillinase sensitive penicillins except ampicillin and carbinicillin. Results may be applied to phenoxymethyl penicillin and phenethicillin.

7. Any of the commercially available 300 or 250 mcg sulfonamide discs can be used with the same standards of zone interpretation. Blood-containing media are not satisfactory for testing sulfonamides.

8. Of the tetracyclines only tetracycline is tested and the results may be applied to chlortetracycline, demeclocycline, doxycycline, methacycline, oxytetracycline, minocycline, and rolitetracycline.

Comments.

1. Individual containers of sensitivity discs should be refrigerated at 4-5°C or frozen at -20°C or below until needed. Discs containing drugs belonging to the penicillin family or cephalosporins should always be kept frozen to maintain their potency except for a small working supply which can be refrigerated for up to one week. New containers of sensitivity discs should be allowed to reach room temperature before being opened. Dispensers containing sensitivity discs should be stored with a dessicant in the refrigerator and the discs allowed to warm to room temperature before being used. Discard any leftover discs on the manufacturers stated expiration date.

2. Use only those drug potencies recommended and when possible perform tests on pure culture isolates. Extremes in inoculum should be avoided and the inoculum should be standardized as described. Undiluted overnight broth cultures should never be used.

3. Diffusion techniques have been standardized with rapidly growing pathogens, such as staphylococci and Enterobacteriaceae, and cannot be reliably applied to slow growing organisms which may show much larger zone sizes than rapid gorwers. Therefore, susceptibility tests on organisms which are fastidious in their nutritional requirements, require an anaerobic atmosphere or increased concentration of CO_2 for growth, or demonstrate an unusually slow growth rate should be determined by the agar dilution method pending development and standardization of suitable diffusion tests. Studies by Bennett, et.al.,[15] in 1968 indicated that a slight modification of the recommended method may be applicable for detecting sulfonamide resistance of Neisseria meningitidis.

A multiply sensitive control strain of Staphylococcus aureus (ATCC 25923) should be run with the gram positive set of discs and a multiply sensitive control strain of Escherichia coli (ATCC 24922) with the gram negative set of discs. A control strain of Pseudomonas aeruginosa (27853) should be tested with carbinicillin, chloramphenicol, colistin, sulfa, gentamicin, kanamycin, tetracycline, and tobramycin.

1. The control strains should be tested daily or at least each time tests are conducted and the zone sizes recorded on a quality control chart for each drug or antibiotic.

2. The zone diameters for the control organisms should fall into the ranges indicated in the table on the following page. Variations on either side of the control limits must be investigated and corrected to insure valid susceptibility test results.

More detailed information on susceptibility testing may be found in the National Committee for Clinical Laboratory Standards publication.[16]

Table II

SUSCEPTIBILITY OF CONTROL STRAINS

tibiotic	Disc potency	Zone diameter of inhibition (mm) S. aureus (ATCC 25923)	E. coli (ATCC 25922)	P. aeruginosa (ATCC 27853)
picillin	10 mcg	24-35	15-20	-----
citracin	10 U	17-22	-----	-----
rbenicillin	100 mcg	-----	24-29	20-24
phalothin	30 mcg	25-37	18-23	-----
loramphenicol	30 mcg	19-26	21-27	6
indamycin	2 mcg	23-29	-----	-----
listin	10 mcg	-----	11-15	12-16
ythromycin	15 mcg	23-30	8-14	-----
ntrisin	250 or 300 mcg	-----	22-26	6
ntamicin	10 mcg	19-27	19-26	16-21
namycin	30 mcg	19-26	17-25	6
thicillin	5 mcg	17-22	-----	-----
lidixic acid	30 mcg	-----	21-25	-----
omycin	30 mcg	18-26	17-23	-----
trofurantoin	300 mcg	-----	20-24	-----
nicillin G	10 U	26-37	-----	-----
lymixin B	300 U	7-13	12-16	-----
reptomycin	10 mcg	14-22	12-20	-----
tracycline	30 mcg	19-28	18-25	9-14
imethoprim - sulfamethoxazole	1.25 mcg 32.75 mcg	24-32	24-32	-----
bramycin	10 mcg	19-29	18-26	19-25
ncomycin	30 mcg	15-19	-----	-----

Table III

PROPOSED DISCS FOR ROUTINE USE[1]

AM POSITIVES[2]	GRAM NEGATIVES[3]
Cephalothin - 30 mcg	1. Ampicillin - 10 mcg (Results do not apply to other penicillins)
Chloramphenicol - 30 mcg	2. Cephalothin - 30 mcg
Clindamycin - 2 mcg	3. Chloramphenicol - 30 mcg
Erythromycin - 15 mcg	4. Kanamycin - 30 mcg
Kanamycin - 30 mcg	5. Gentamicin - 10 mcg
Methicillin - 5 mcg (Results apply to other penicillinase resistant semisynthetic penicillins)	6. Colistin - 10 mcg (Results apply to polymyxin B)
Gentamicin - 10 mcg	7. Carbinicillin - 100 mcg
Penicillin G - 10 Units (Results apply to ampicillin)	8. Tobramycin - 10 mcg
Tetracycline - 30 mcg (Results apply to analogues)	9. Trimethoprim - sulfamethoxazole 1.25 mcg/32.75 mcg
Vancomycin - 30 mcg	10. Tetracycline - 30 mcg (Results apply to analogues)
Optional (Sulfas, ampicillin)	11. Nitrofurantoin[4] - 300 mcg
Optional[5] (Neomycin, Bacitracin)	12. Nalidixic acid[4] - 30 mcg

e use of 12 high content discs on one plate requires careful placement of tibiotic discs to avoid overlapping zones.

aphylococcus, Streptococcus, enterococci, and aerobic gram positive rods. terococci should be tested with the ampicillin disc.

coli, Klebsiella, Enterobacter, Proteus, Pseudomonas, etc.

r isolates from urinary tract infections only.

r topical use only.

tional spaces have been left open for special requests of clinicians for sceptibility tests, or for any additional antibiotics that your laboratory ght wish to use in different situations.

POUR-STREAK PLATES

From throat swabs

1. Place throat swab in 1 ml of broth and incubate at 37°C for 2 hrs. Specimens received in the laboratory within 2-4 hours of taking may be cultured with 0-2 hours incubation in broth depending on the available time remaining in the working day.

   Specimens which have been in transit 4-8 hours should have a minimum of 2 hours incubation in broth while those in transit over 8 hours should have 4-5 hours incubation in broth.

2. Remove the swab from the 1 ml of broth, drain against inside of tube and place in a sterile tube.

3. Take a tube with 15-20 ml of blood agar base, melted and held in a water bath at 48-50°C. Cool to approximately 45°C before use.

4. Add 0.6 - 0.8 ml of sterile defibrinated blood to melted and cooled agar.

5. From the broth with the swab washings (step 3), transfer one drained loop to the blood agar tube.

6. Mix thoroughly, flame lip and pour into a sterile Petri dish.

7. Wipe lip of tube with disinfectant-soaked cotton and place in discard pans.

8. When the agar is hard, rotate the swab over a small section of the surface. Using an inoculating loop, spread this inoculum over half the plate, cross hatching for isolation. Stab into agar after each cross-hatch series.

From broth cultures

1. Rotate the broth culture to obtain an even suspension of organisms.

2. Transfer one loopful of broth culture to 15 ml of sterile saline and mix well. (If the growth is sparse in the broth it may be necessary to use 2-3 loopfuls.)

3. Add 0.6 - 0.8 ml of sterile defibrinated blood to 15-20 ml of blood agar base, melted and cooled to 45°C.

4. Transfer one loopful of the saline suspension to the agar blood mixture.

5. Mix thoroughly, flame lip and pour into a sterile Petri plate.

6. Wipe lip of tube with disinfectant-soaked cotton and place in discard pans.

7. When the agar is hard, transfer one loopful of the broth culture to the surface of the agar and streak over half the plate, cross hatching for isolation.

PICKING BETA HEMOLYTIC COLONIES FROM POUR-STREAK PLATES AND INOCULATING "PIE" PLATES

1. Examine plate macroscopically for beta hemolytic colonies and mark typical surface colonies. Pick typical surface colonies.

2. Examine sub-surface colonies microscopically with low-power objective or dissecting microscope and mark colonies with typical beta hemolysis. To pick sub-surface colonies, steady hand by resting forearm on bench, then stab needle vertically through agar into colony. Move needle point sufficiently to contact entire colony, then withdraw needle.

3. With each colony picked, streak surface of blood agar plate in a "pie"-shaped area, so that isolated colonies are obtained.

4. Incubate plates overnight at 35-37°C.

IDENTIFICATION OF GROUP A STREPTOCOCCI BY MEANS OF THE FLUORESCENT ANTIBODY TECHNIQUE

ɐparation and fixing of smear:

1. Place throat swabs in 1 ml broth and incubate for 2-5 hours at 37°C.
2. Remove swab from broth and place in sterile tube.
3. Centrifuge broth for 5 minutes at approximately 2,000 rpm.
4. Pour off broth into disinfectant solution and wipe lip with disinfectant-soaked cotton; resuspend cells in 1 ml sterile buffered saline pH 7.6 and re-centrifuge.
5. Pour off buffered saline into disinfectant solution. Wipe lip with disinfectant-soaked cotton while tube is in inverted position to remove all visible diluent.
6. Place tube in rack for 2-3 minutes and let residual buffered saline collect in bottom of tube. (Usually no additional diluent will be necessary).
7. Mix cells thoroughly in diluent.
8. With a Pasteur capillary pipette transfer sediment to area within circles on F.A. slide. An attempt should be made to transfer most of the sediment to the smears.
9. Let smears dry in air. If atmosphere is humid, drying may be done in 37°C incubator.
10. Cover each smear with 95% ethanol. Keep wet for 1 minute, then let ethanol evaporate. After smears are thoroughly dry, they may be stained or may be frozen and stored and stained at a later date if absolutely no thawing occurs in the interim.

aining:

Apply procedure to thoroughly dried, fixed smears. Include a known positive control slide in each test run.

11. Cover smear nearest etched end of slide with small drop of Control Conjugate (normal rabbit globulin labeled with fluorescein-isothiocyanate and treated to remove non-specific staining.)*

* After the desirable sensitivity and specificity of a particular vial of Group A antistreptococcus conjugate has been established with Groups A, C, and G streptococcus and Staphylococcus aureus control cultures, it is permissable to omit staining with the normal rabbit globulin Control Conjugate routinely.

Cover the other smear with Group A Antistreptococcus Conjugate (Group A antistreptococcus globulin labeled with fluorescein-isothiocyanate and treated to remove non-specific staining).

Spread each conjugate over entire smear with an applicator stick held in a horizontal position.

12. Let stand 30 minutes at room temperature in a moist chamber. Half a 15 cm Petri dish fitted with moist filter paper makes a suitable chamber.

13. Shake excess conjugate onto disinfectant-soaked paper towel.

14. Dip slides momentarily into buffered saline pH 7.6 in a staining dish.

15. Transfer to a second vessel of buffered saline, and let stand for 10 minutes.

16. Dip momentarily into distilled water and air dry.

17. Add a drop of buffered glycerol mounting fluid and a cover slip. Avoid formation of bubbles in mounting fluid. Apply one drop of nail polish (or similar adhesive) to each cover slip corner.

18. Stained and mounted smears may be refrigerated (not frozen) and examined anytime within 24-48 hours without significant loss of brilliance of fluorescence. Smears may be stored in the refrigerator for longer periods to be used later for reference purposes. In these there may be gradual loss of staining intensity. Slides to be stored in the refrigerator overnight or longer should be sealed completely with nail polish.

19. Recommended filter system:

BG12 Exciter OG1 Barrier

Estimation of Fluorescence - Intensity of Stained Cells:

4+ : Maximal fluorescence, brilliant yellow-green; clear-cut cell outline; sharply defined non-staining center of cell.

3+ : Less brilliant yellow-green fluorescence; clear-cut cell outline; sharply defined non-staining center of cell.

2+ : Less brilliant, but definite fluorescence; less clear-cut cell outline; non-staining center area fuzzy.

1+ : Definite fluorescence, but very subdued; peripheral and center staining of same intensity.

PREPARATION OF ACID-HEAT EXTRACT FOR PRECIPITIN GROUPING OF BETA HEMOLYTIC STREPTOCOCCI:

1. From 30 ml broth culture make a blood agar streak plate and a smear for Gram's stain. Examine the smear to be sure you have a pure culture of streptococci. (Staining and examination of smear may be done between steps 2 and 3.)

2. Discard the cotton plug in a pan of disinfectant and centrifuge at 2000 RPM for 30 minutes. Label clearly.

3. After 30 minute centrifugation, pour the supernatant fluid into a jar of disinfectant and wipe lip of tube with disinfectant-soaked cotton.

4. To the sediment add one drop of 0.04% meta-cresol-purple and 0.3 ml of N/5 HCl in physiological saline. Mix well with a Pasteur capillary and transfer to a Kahn tube. If the suspension is not a definite pink color (pH 2.0 - 2.4) add another drop or two of N/5 HCl. Label clearly.

5. Place in a boiling water bath for 10 minutes, shaking several times.

6. Remove from water bath, relabel and centrifuge 30 minutes.

7. The supernatant fluid is the extract. Decant into another Kahn tube and discard the sediment.

8. Neutralize the extract by adding N/5 NaOH (in distilled H_2O) drop by drop until it is slightly purple, (pH 7.4 - 7.8). A deep purple color indicates that too much NaOH has been added. Adjustment back to light purple may be made with N/20 HCl (in distilled H_2O) but this dilution should be avoided if possible.

9. Label clearly and centrifuge for 10 minutes.

10. Decant the supernatant fluid into a small screw-cap vial. Label carefully and store in refrigerator.

Meta Cresol Purple:

200 mg powder - grind in mortar with 26.7 ml of N/50 NaOH.

Dilute above mixture to 500 ml with distilled H_2O for working solution.

PREPARATION OF MAXTED ENZYME EXTRACT FOR PRECIPITIN GROUPING OF BETA HEMOLYTIC STREPTOCOCCI

1. Pipette 0.25 ml of enzyme solution* into a small test tube (12 x 75 mm or smaller).

2. Suspend in this solution a small amount of growth from a 16-24 hour blood agar plate culture of beta hemolytic streptococci.

3. Place in 45°C water bath until solution is clear (about 1½ hours).

4. Cool to room temperature and centrifuge for 10 minutes at 2000 RPM.

5. Perform precipitin grouping as with acid-heat extracts.

This procedure yields satisfactory extracts of beta hemolytic streptococci commonly isolated from human sources with the exception of Group D strains.

*Streptomyces albus enzyme; available commercially.

PRECIPITIN GROUPING OF BETA HEMOLYTIC STREPTOCOCCI

1. Dip capillary* into serum (in screw-cap vial) until a column about 1 cm long has been drawn in by capillary action. (To maintain sterility of the sera the capillaries are sterilized and should be kept sterile at the lower end until after the serum is taken up.)

2. Wipe off capillary with facial tissue taking care to hold tube so air does not enter the end.

3. Dip capillary into extract until an amount is drawn up equal to the serum column. If an air bubble separates serum and extract, discard capillary and repeat.

4. Wipe capillary carefully. Fingerprints, serum or extract on the outside of the capillary may simulate or obscure a positive reaction.

5. Plunge the lower end of the capillary into the plasticine until a small plug fills the opening.

6. Invert capillary and insert gently into the plasticine-filled groove of the rack.

7. After 10 minutes, examine with a bright light against a dark background. A white cloud or ring at the center of the column represents a positive test. A strong reaction appears in 5-10 minutes; a weaker reaction develops more slowly. Since after 30 minutes the reaction may fade or a false positive appear, it is important to examine the tubes at frequent intervals between 10 and 30 minutes.

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* Vaccine capillary tubes, Kimble borosilicate glass, both ends open and lightly fire-polished. Tubes to be bulk packed. O.D. 1.20 to 2 mm. (in 50 lb. lots).

NEUFELD QUELLING TEST

1. Place drop of the material to be tested (spinal fluid, sputum, etc.), or of a light suspension of a young culture on a slide.

2. Near it place a drop of methylene blue.

3. At a third spot, near the others, place a drop of specific antiserum.

4. Using a sterile loop or applicator stick, mix stain and organism suspension; then mix serum into the drop of stained organisms.

5. Cover with a #1 cover slip and examine with oil immersion objective after 10-15 minutes. If negative, reexamine after another 10-15 minutes.

6. In the presence of specific antiserum the capsule of the organism appears to be considerably larger than in a preparation with an heterologous antiserum.

Organisms which have specific capsular polysaccharides (pneumococci, meningococci, H. influenzae, etc.) may be identified by this procedure.

The test may be done on body fluids such as spinal fluid, sputum, etc. and hence permits rapid, specific diagnosis. It may also be done on young cultures from broth or solid media. However if cultures are used care must be taken to have a suspension of less than 50 organisms per oil-immersion field. Larger numbers of organisms may dissipate the antibody to such an extent that capsular swelling is not apparent.

OPTOCHIN DISC SENSITIVITY TEST FOR

S. PNEUMONIAE

1. Streak the surface of half of a blood agar plate with a suspected colony of the pneumococcus.

2. With flamed forceps, place an optochin disc in the center of the inoculated area.

3. Incubate at 37°C for 18-24 hours.

4. Measure the zone of inhibition in millimeters including the diameter of the disc.

   a. 6 mm disc:

      + = zone of inhibition 14 mm or greater.
      - = no zone of inhibition.
      doubtful = any zone of inhibition less than 16 mm.

   b. 10 mm disc:

      + = zone of inhibition 16 mm or greater.
      - = no zone of inhibition.
      doubtful = any zone of inhibition less than 16 mm.

   c. Typical streptococci and a positive optochin test is indicative of S. pneumoniae. Cultures giving a doubtful optochin test may be alpha hemolytic streptococci or S. pneumoniae. These cultures should be tested by the bile solubility test, Quellung test, and/or the agglutination tests.

SOLUBILITY TEST FOR S. PNEUMONIAE

1. Use 24-48 hour broth culture. The broth should contain 0.5% dextrose to support adequate growth of the pneumococci.

2. Prepare a Gram stain to check for purity.

3. Add one drop of phenol red indicator to broth culture.

4. If acid, neutralize with 2-3 drops of N/1 NaOH. (In acid medium sodium desoxycholate may form a precipitate and/or gel which interferes with the reading of the test.)

5. Place 0.5 ml of the neutralized broth culture in each of two small, clear test tubes (12 x 75 mm).

6. To one tube add 0.5 ml of 2% sodium desoxycholate.

7. To the other tube add 0.5 ml of physiological saline.

8. Place in 37°C incubator for 10 minutes.
   If the tube with sodium desoxycholate has not cleared, re-incubate and examine at intervals for up to one hour.

Alternate method

1. Scrape some of the growth from a blood agar plate into 1-2 ml of physiological saline.

2. Shake well or mix with a pipette to obtain a smooth suspension.

3. Proceed with test as described above.

S. pneumoniae is readily soluble in desoxycholate whereas streptococci are not.

SLIDE TEST FOR CLUMPING FACTOR OF STAPHYLOCOCCI

1. On a slide, emulsify the organisms from a single Staphylococcus-like colony in a small drop of physiological saline. The suspension must be quite heavy.

2. With an inoculating loop mix into the emulsion a small drop of coagulase plasma.

3. Read within 5 seconds. Strains showing clumping within 5 seconds may be recorded as coagulase positive. If no clumping occurs a coagulase test must be done.

This test may prove useful as a screening test for large numbers of cultures from carriers or environment. It should be emphasized, however, that appreciable numbers of coagulase positive staphylococci are slide test negative.

Do not attempt to do this test on colonies from mannitol salt or other selective, inhibitory media.

COAGULASE TEST FOR S. AUREUS

1. Prepare and examine a Gram stain preparation.

2. Dispense plasma in 0.5 ml amounts to sterile 12 x 75 mm tubes.

3. Using a Pasteur capillary pipette add 2 drops of a broth culture to 0.5 ml of coagulase plasma.

4. Leave one tube of coagulase plasma uninoculated as negative control.

5. Incubate tubes in an incubator or a water bath at 35-37°C for 4 hours.

6. Examine for clotting, any degree being considered positive.

Many coagulase positive strains will clot plasma in one hour. A very few will develop a clot only after more than 4 hours incubation. If sterile coagulase plasma is used final readings may be made after 24 hours incubation if desired.

Warning: Desiccated commercial coagulase plasmas are not prepared under aseptic conditions and hence may not be sterile. Therefore, incubation beyond the recommended 4 hour period may give erroneous results.

If fresh human or rabbit plasma from blood bank or other sources is used, each batch must be tested with 3-4 strains of staphylococci whose coagulase activity is known. Included among the test strains should be at least one strongly positive, one weakly positive, and one negative.

DETERMINATION OF X AND V FACTOR REQUIREMENTS OF *HAEMOPHILUS*

1. Make a suspension of cells in sterile distilled water or saline.

2. Dip a sterile swab in the organism suspension. Roll the swab over the entire surface of a heart infusion agar plate.

3. With sterile forceps, place X, V, and XV discs* or strips at equal distances apart on the inoculated plate.

4. Incubate at 37°C overnight.

5. Observe for growth immediately around the discs or strips.

*Certain of the commercially available discs contain bacitracin. Since organisms *other than Haemophilus* are inhibited by bacitracin, these organisms do not grow in the area immediately surrounding the disc, but growth may occur farther away from the disc. Such results are not typical of *Haemophilus* which grows only close to the disc.

AMPICILLIN RESISTANCE OF HEMOPHILUS INFLUENZAE

A. Detection of beta lactamase.[17]

1. Prepare test penicillin substrate solution.

   a. Add 2 ml of a 0.5% phenol red solution to 16.6 ml of sterile distilled water. Add this solution to a vial containing 20 million units of potassium penicillin G (Pfizer).

   b. Add 1 M sodium hydroxide drop by drop until the test solution turns violet (pH 8.5).

   c. The test solution may be used immediately or may be divided into portions in screwcap tubes and be frozen at -60° to -70°C for up to 1 week.

2. Microtiter plate method.

   a. Add 0.1 ml test penicillin substrate to the well of a microtiter plate for each culture to be tested.

   b. Using a loop, scrape the growth equivalent to one colony off of a 24 hour chocolate agar plate of H. influenzae and suspend this growth in the substrate.

   c. Let plate stand at room temperature for 15 minutes. The development of a yellow color in the substrate indicates a beta lactamase positive organism. No color change or a pale pink color is a negative test.

3. Capillary tube method.

   a. Dip a capillary tube (0.7 - 1.0 mm OD) into the penicillin test substrate and take up 1-2 cm of liquid.

   b. Scrape the tip of the capillary lightly across several colonies of H. influenzae on a 24 hour chocolate agar plate so that a plug of bacteria fills the bottom of the tube. Allow no air to be trapped between the test substrate and the bacteria since the two must come in contact.

   c. Invert the capillary and let it stand for 15 minutes at room temperature. The development of a yellow color in the substrate indicates a beta lactamase positive organism as in the microtiter plate method.

. Antibiotic disc susceptibility method.

1. Using a loop, suspend the growth from a 24 hour chocolate agar plate of H. influenzae in Mueller-Hinton broth to obtain a turbidity equivalent to a number 0.5 MacFarland standard (approximately 10^8 organisms/ml). See page 114 for preparation of the standard.

2. Using a cotton swab, inoculate a Mueller-Hinton chocolate agar plate with the test suspension as described on page 115 for the disc susceptibility test. The plate must be prepared by adding 1% hemoglobin and 1% isovitalex (BBL) to Mueller-Hinton agar base. It is satisfactory to use 20 ml of medium in a 100 mm Petri plate and 2-3 cultures may be tested on each plate by swabing each culture over a portion of the plate.

3. Place a 10 mcg ampicillin disc on the inoculum and incubate the plates for 24 hours at 35°C without added CO_2.

4. After 24 hours incubation, read the zone size around each disc under reflected light. A zone size of 20 mm or greater indicates an ampicillin susceptible strain. A zone size less than 20 mm indicates an ampicillin resistant strain.

CHOCOLATE AGAR FOR NEISSERIA

A. G-C chocolate agar.

1. G-C Medium Base (Difco) or GC agar base (BBL) 7.2 gm.
   Distilled water, cold 100 ml.

   Dissolve by heating at 100°C a few minutes.
   Mix well and autoclave at 121°C for 15 minutes.

2. Hemoglobin 2.0 gm.
   Distilled water 100.0 ml.

   Add hemoglobin to 25-50 ml of the water in a flask containing a layer of glass beads. Shake vigorously to dissolve the hemoglobin and then add remainder of water. Filter through coarse, moistened cheesecloth to remove any undissolved particles and autoclave at 121°C for 15 minutes.

3. Cool 1 and 2 to 50-56°C. Mix in equal quantities (both solutions being double strength) under aseptic conditions and add 1% Bacto supplement B or isovitalex (BBL) enrichment. Dispense aseptically to plates or slants.

B. Thayer-Martin medium.

1. Prepare G-C chocolate agar as in part A.

2. For each 200 ml of media containing the enrichment at 50-56°C, add 2 ml vancomycin-colistin-nystatin (V-C-N) inhibitor (BBL) or 2 ml colistimethate-nystatin-vancomycin (C-N-V) inhibitor (Difco). Mix and dispense aseptically to plates.

OXIDASE TEST

Reagent

Tetramethyl-p-phenylene-diamine hydrochloride	0.5 gm.
Distilled water	100 ml.

This solution is good for 1 week if stored in the refrigerator at 4-10°C.

For routine use in small amounts it is convenient to prepare it in the following manner:

Weigh carefully 50 mgm of the powder and transfer to a 12 x 75 mm cotton plugged tube. Prepare 10 or 12 tubes and store in a brown screw top bottle with some calcium sulfate in the bottom. For use add 10 ml distilled water to one tube and transfer to a small dropper bottle.

Test procedure

Add a few drops of the oxidase reagent to suspected colonies on a plate. Colonies of oxidase positive organisms undergo a color change through dark blue to black. The reagent will kill the organisms and any subculture of colonies must be made as soon as possible after the color change begins to occur. Read for color change up to 1 minute.

Kovac's modification

Place 3-4 drops of oxidase reagent on a piece of filter paper. Using a loop, immediately mix a loopful of the organisms from a plate or slant into the reagent. The color change occurs in a positive test as in the routine procedure. Read for color change within 10 seconds.

NEISSERIA FERMENTATION TESTS

A. The modified rapid fermentation test reported by Brown[18] is a satisfactory procedure for obtaining rapid tentative identification of Neisseria isolated from clinical specimens. The tentative identification should be confirmed by testing the same sugars in cystine trypticase agar (CTA).

B. Modified rapid fermentation test.

1. Prepare pH 7.0 buffer-salt solution (BSS).

   a. Dissolve:

Dipotassium hydrogen phosphate (K_2HPO_4)	0.04 gm
Potassium dihydrogen phosphate (KH_2PO_4)	0.01 gm
Potassium chloride (KCl)	0.8 gm
Distilled water	100.0 ml
Phenol red (1% aqueous solution)	0.2 ml

   b. This solution may be stored at 5°C in a sterile screwcap bottle for up to 8 weeks.

2. Prepare 20% solutions of glucose, lactose, sucrose, and maltose. These solutions may be stored in 5 to 10 ml amounts at -20°C for up to 6 months. Thawed solutions may be stored at 5°C for up to 8 weeks. Check visually for contamination before each use.

3. For each culture to be tested, place 0.3 ml of BSS in a 10 x 75 mm tube and add two 4-mm loopfuls of an actively growing 24 hour pure culture of Neisseria. Mix well.

4. Label one 10 x 75 mm test tube each for glucose, lactose, sucrose and maltose.

   a. Add 0.1 ml BSS to each tube.

   b. With a Pasteur pipette, add one drop of carbohydrate solution to its labeled fermentation tube.

   c. With a Pasteur pipette, add one drop of the cell suspension to each fermentation tube.

   d. Shake the tubes gently and incubate in a 37°C waterbath up to 4 hours.

   e. Read the tubes for development of a yellow color indicating fermentation of the carbohydrate.

C. Fermentation in CTA media.

1. Prepare 20% solutions of glucose, lactose, sucrose, and maltose in distilled water and sterilize by Seitz or other type of filtration.

2. Prepare dehydrated CTA medium containing phenol red indicator, adjust pH to 7.6, and sterilize by autoclaving. Cool to 56°C and add 5 ml of 20% carbohydrate solution to each 100 ml of media. Tube in 7 ml amounts in 16 x 125 mm screwcap tubes. Store at 5°C.

3. Inoculate CTA media with a heavy inoculum using a loopful of inoculum from an actively growing 24 hour culture of Neisseria. Stab each of the fermentation media 4 times to a depth of about 1/2 inch.

4. Incubate the tubes with the caps loose for 48 hours. Observe the tubes at 24 and 48 hours for the development of a yellow color at the top of the tubes indicating fermentation of the carbohydrate tested.

	Branhamella (Neisseria) catarrhalis	Neisseria flavescens	Neisseria gonorrhoeae	Neisseria meningitidis	Neisseria* subflava	Neisseria lactamica	Neisseria* flava	Neisseria* perflava	Neisseria sicca	Neisseria mucosa
Number of strains	49	5	180	2000	23	289	26	98	56	36
Oxidase	+	+	+	+	+	+	+	+	+	+
Carbohydrates in CTA base										
Glucose	-	-	A(-)	A(-)	A(-)	A	A(-)	A(-)	A(-)	A(-)
Lactose	-	-	-	-	-	A	-	-	-	-
Sucrose	-	-	-	-	-	-	-	A	A	A
Maltose	-	-	-	A(-)	A	A	A	A	A	A
Fructose	-	-	-	-	-	-	A	A	A	A
Growth on:										
Nutrient agar 35°C	+(-)	+	-	-(+)	+	+(-)	+(-)	+(-)	+	+
Nutrient agar 25°C	+(-)	+	-	-	+	-(+)	+(-)	+(-)	+	+
Thayer-Martin	-	-	+	+	-	+	-	-	-	-
Nitrate, routine	+(-)	-	-	-	-	-	-	-	-	+ gas
Pigmentation on Loefflers	- (slight yellow)	yellow (slight yellow)	-	- (slight yellow)	yellow (slight yellow)	slight yellow (-)	yellow (slight yellow)	yellow (slight yellow)	- (slight yellow)	- (slight yellow)

*In the 8th edition of Bergey's manual all three species are called N. subflava.

BIOCHEMICAL SET FOR GRAM NEGATIVE RODS

Medium	Inoculum	Amount
TSI slant (add lead acetate paper)	HIA slant*	Stab to bottom and streak slant.
Sugars: Glucose, Xylose, Mannitol, Lactose, Sucrose, Maltose		Stab 4 X 1/2 in. below surface.
Motility		Stab once 1/2 in. below surface.
Simmons' citrate		Streak lightly on slant.
Blood agar plate	HIB**	Loopful. Streak.
MacConkey slant		One drop.
SS slant		
Nitrate broth		
Tech.		
Flo.		
Tryptone broth for indole		
Christensen's urea slant		
HIA slant (for catalase)		
Gelatin		4 drops
Litmus Milk		" "

* 18-24 hour heart infusion agar slant.

** 18-24 hour heart infusion broth culture.

OXIDATION VERSUS FERMENTATION OF CARBOHYDRATES

Bacteria produce acid from carbohydrates by two methods. One is an anaerobic process called fermentation; the second, designated oxidation, is an aerobic process. Since gram negative bacteria which attack carbohydrates usually do so exclusively by either fermentation or oxidation, the determination of the type of carbohydrate metabolism carried out by an organism is of taxonomic significance.

Enteric base carbohydrate media will detect acid production by fermentative organisms. Oxidative organisms do not usually produce a detectable amount of acid in enteric media since the acidity produced by oxidation is less than by fermentation and because sufficient alkaline products are frequently produced from the peptone in the medium to neutralize the acid produced by oxidative metabolism.

Triple sugar iron agar (TSI) can be used to differentiate fermentative from oxidative bacteria. A TSI reaction of acid butt with or without an acid slant is indicative of a fermenter. No change of the butt and slant of a TSI or slight alkalinization of the slant is indicative of an oxidizer or non-utilizer of sugars. Occasionally an oxidizer will show slight acidity on the slant of a TSI. In cases where the TSI reaction is doubtful, an oxidation-fermentation test can be carried out using OF base medium with 1% glucose as described on page 143.

An OF base medium should be used to determine the pattern of carbohydrate metabolism of oxidative bacteria. OF base media contain less peptone than do the fermentation bases and 0.3% agar. The agar prevents convection currents and allows the acid produced to remain concentrated in the medium adjacent to where it is produced and not be diluted by mixing throughout the medium.

OXIDATION-FERMENTATION MEDIA AND REACTIONS

A. Oxidation carbohydrate base (for oxidizers).

1. Medium.

a. O-F basal medium (Difco). This medium contains bromthymol blue indicator.

Basal media without carbohydrate: adjust to pH 7.0. Tube in 15 x 125 mm tubes, cotton plugs, 6.0 ml per tube. Autoclave 15 lbs., 15 minutes. While base is melted, add Seitz filtered carbohydrate to a final dilution of 1%, (i.e., 0.6 ml of a 10% carbohydrate solution to 6.0 ml of base). Twirl to mix and allow to solidify; do not slant. Do not add the carbohydrate before autoclaving.

2. Test procedure.

a. Inoculate tubes of medium with a needle by stabbing 4 times approximately 1/2" below the surface with growth from a young infusion agar slant culture.

b. Incubate at 35-37°C and observe daily for 7 days for acid production.

B. O-F test.

1. To determine the oxidation-fermentation characteristics of a pure culture inoculate 2 tubes of O-F basal medium containing 1% dextrose. Overlay 1 of the tubes with sterile petrolatum jelly. Incubate at 35-37°C.

2. Acid formation in the open tube only indicates oxidative utilization of dextrose. Acid formation in both the open and closed tubes indicates fermentative utilization of dextrose. No acid in either tube indicates the non-utilization of dextrose.

C. Fermentation carbohydrate base (for fermenters).

Peptone	10.0 g
Meat extract	3.0 g
Sodium chloride	5.0 g
Andrade's indicator*	10.0 ml
Distilled water	1000.0 ml

Adjust pH to 7.2. Tube in 6 ml amounts in 15 x 125 mm tubes with inverted insert vials and sterilize at 121 C for 15 minutes.

Glucose, lactose, sucrose, and mannitol are employed at a final concentration of approximately 1% by adding 0.6 ml of a 10% sterile solution of the carbohydrate to the base medium. Other carbohydrates are employed at a final concentration of approximately 0.5% by adding 0.6 ml of a 5% sterile solution of the carbohydrate.

*Andrade's indicator.

Distilled water	1000.0 ml
Acid fuchsin	5.0 gm
Sodium hydroxide (1N)	160.0 ml

Dissolve the fuchsin in distilled water and add the sodium hydroxide. If, after several hours, the fuchsin is not sufficiently decolorized, add an additional 1 to 2 ml of alkali. Each lot of acid fuchsin dye varies in dye content and the amount of alkali to use with any given lot should be specified on the label. The reagent improves somewhat on aging and should be prepared in sufficiently large amounts to last for several years.

REDUCTION OF NITRATES

Medium

Add 0.1% of KNO_3 to a broth medium suitable for the growth of the organism to be tested. At the CDC a 2% peptone medium adjusted to pH 7.0 is used for routine cultures. Heart infusion broth is substituted for more fastidious organisms.

Tube the media in 5 ml amounts in 15 x 125 mm tubes with a gas insert vial. Autoclave at 121°C for 15 minutes.

Nitrite Test Reagents

Solution #1

Glacial acetic acid	100.0 ml.
Distilled water	250.0 ml.
Sulfanilic acid	2.8 gm.

Solution #2

Glacial acetic acid	100.0 ml.
Distilled water	250.0 ml.
Dimethyl-alpha-naphthylamine	2.1 ml.

Procedure

1. To 48 hour nitrate broth culture add:

   a. 5 drops nitrite reagent #1

   b. 5 drops nitrite reagent #2

2. Observe for red color developing within 1-2 minutes.

   The red color indicates presence of nitrites. If no red color develops it may mean that 1) the nitrate in the medium has not been reduced, or 2) it has been reduced beyond the nitrite stage to some other compound or to nitrogen as detected in the gas insert vials. To determine which has taken place add a little powdered zinc to the tube and shake vigorously. If nitrates are still present they will be reduced by the zinc and a red color will develop in 5-10 minutes.

MOTILITY TESTING

Semi-solid agar method:

Medium - Motility medium (BBL) containing a 0.4% final concentration of agar or equivalent such as Difco motility medium modified as follows:

Difco motility medium	16 gm
Nutrient broth	4 gm
NaCl	1 gm
Distilled water	1000 ml

Tube 4 ml in 13 x 100 test tubes and autoclave at 121°C for 15 minutes.

Procedure - Take a small amount of growth on a straight needle from an 18-24 hour infusion agar slant culture. Inoculate once to a depth of only 1/4 to 1/2 inch in the middle of the tube. Incubate at 35 to 37°C and examine daily up to 7 days. Motile organisms will spread out into the medium from the site of inoculation.

Hanging Drop Method:

Use a young (6-24 hour) actively growing broth culture. Place one loopful of the culture in the center of a #1 cover slip. On each corner of the cover slip put a small drop of immersion oil. Invert the cover slip over the concavity of a depression slide. Examine with the high dry (4 mm) objective if possible. If this magnification is inadequate, use oil immersion, taking care not to crack the cover slip.

In a hanging drop preparation motility must be differentiated from Brownian movement and flow of the fluid caused by pressure on the cover slip. In true motility the organisms change position with respect to each other whereas in Brownian movement and fluid flow they may appear quite active but remain in the same relative position to other organisms or debris in the field.

PIGMENT PRODUCTION

"Flo" and "Tech" pigment media were developed to enhance formation of water soluble pigments fluorescin and pyocyanin. (Ref: J. Lab. and Clin. Med., 44,301-307 1954)

"Tech" (Medium P)		"Flo" (Medium F)	
Bacto-peptone	20 gms	Proteose peptone #3	20 gms
Bacto-agar	15 gms	Bacto-agar	15 gms
Glycerol CP	10 ml	Glycerol CP	10 ml
$MgCl_2$ (anhyd.) CP	1.4 gm	K_2HPO_4 (anhyd.)	1.5 gms
K_2SO_4 (anhyd.) CP	10 gms	$MgSO_4$ CP	1.5 gms

Adjust to pH 7.2. Dispense in quantities sufficient to give a generous slant and butt (7 ml in 15 x 125 mm tubes). Sterilize by autoclaving at 121°C for 15 minutes. (The correct peptones are most important.)

Procedure:

Inoculate surface of slants from agar or broth culture. Do not inoculate butt of agar (these pigments are colorless in an anaerobic atmosphere).

Incubate at 35-37°C. Examine after 24 hours and up to 7 days. Pigment will usually be seen by 72 hours.

Fluorescin may be demonstrated by ultra violet light* in a darkened room. This type of illumination is not required for the other pigments. (Fluorescin may be produced in greater amount after incubation at 25°C.)

Results:

"Tech" (Medium P): Colors appearing in this medium vary with the strains studied. Predominantly pyocyanin-producing strains of Ps. aeruginosa give pigment ranging from light aqua to dark blue or green. With strains producing mainly pyorubrin the color ranges from light pink to dark maroon. Strains producing both pigments show a variety of colors including many shades and mixtures of reds and blues.

"Flo" (Medium F): The pigment observed most frequently on this medium was fluorescent and of a greenish yellow color. Some strains of Ps. aeruginosa also produce a small amount of pyocyanin giving the medium a bright green appearance. Yellowish pigments formed usually after 72 hours which are not fluorescent under UV* are produced by many bacteria and should be ignored. Pseudomonas may also produce a dark brown pigment.

* Wood's lamp is satisfactory.

INDOLE TEST

Media

2% tryptone broth.
Adjust pH to 7.2 - 7.4, tube in 6 ml amounts and autoclave at 121°C for 15 minutes.

Ehrlich's reagent

Ethyl alcohol, 95%	95 ml
Para-dimethylamino-benzaldehyde	1 gm
Hydrochloric acid, conc.	20 ml

Dissolve aldehyde in alcohol and then slowly add acid. The dry aldehyde should be light straw in color. Ehrlich's reagent should be prepared in small quantities and stored in the refrigerator when not in use.

Test procedure using Ehrlich's reagent

1. Incubate tryptone broth culture at 35-37°C for 48 hours.

2. Add 1 ml of xylene to the culture and shake vigorously to extract the indole. Allow to stand 1-2 minutes for the xylene extract to layer on top.

3. Add 0.5 ml of Ehrlich's reagent down the side of the tube. It should form a layer between the broth and the xylene. If indole is present, a red ring will develop just below the xylene layer.

Kovac's reagent

Pure amyl or isoamyl alcohol	150 ml
Para-dimethylamino-benzaldehyde	10 gm
Hydrochloric acid, conc.	50 ml

Mix and note precautions as for Ehrlich's reagent.

Test procedure using Kovac's reagent

1. Add about 0.5 ml of reagent to a 48 hour tryptone broth culture; shake tube gently. A deep red color develops in the presence of indole.

NOTE: Tests for indole production with either Ehrlich's or Kovac's reagents may be made after 24 hour incubation, but all negative tests should be repeated on a separate 48 hour broth culture.

METHYL RED (MR) TEST

Media

Buffered peptone glucose broth (Clark and Lubs formula) - Difco or BBL. The medium selected for MR tests may also be used for Voges-Proskauer (VP) tests.

MR reagent

Methyl red	0.1 gm
Ethyl alcohol, 95%	300.0 ml
Distilled water	200.0 ml

Dissolve dye in the alcohol and then add the water.

Test procedure

1. Incubate broth culture at 35-37°C for 48 hours. Tests should not be made with cultures incubated less than 48 hours. If results are equivocal at 48 hours, the tests should be repeated with cultures incubated at 35-37°C for 4-5 days. In such instances, duplicate tests should be incubated at 25°C.

2. Add 5 or 6 drops of reagent per 5 ml of culture. Reactions are read immediately. Positive tests are bright red, weakly positive tests are red-orange, and negative tests are yellow.

VOGES-PROSKAUER (VP) TEST
(Coblentz Method)

Reagents

1. Reagent A. - 5% alpha naphthol in 95% ethanol. (Keeps one month at room temperature.)

2. Reagent B. - 0.3% creatine in 40% KOH. (Keeps 2 weeks at room temperature. 40% KOH is stable indefinitely.)

Culture

1. Inoculate 2 ml of buffered peptone glucose broth (MR-VP broth) heavily by scraping the growth from an 18-24 hour infusion agar culture with a loop. In the case of organisms which grow lightly, such as Listeria, use a capillary pipette and wash off the entire growth from the slant, using the MR-VP broth as the suspending medium.

2. Incubate the broth culture at 35-37°C for 6 hours.

Test procedure

1. Add 0.6 ml of reagent A to the culture. Shake gently.

2. Add 0.2 ml of reagent B. Shake vigorously.

3. Observe for 5-10 minutes, shaking occasionally.

   Positive = pink to cherry red color.

   Negative = no color change.

VOGES-PROSKAUER (VP) TEST
(O'Meara Method)

Test reagent (O'Meara, modified)

Potassium hydroxide	40 gm
Creatine	0.3 gm
Distilled water	100 ml

Dissolve the alkali in distilled water and add creatine. Store reagent in the refrigerator. Keeps 2 to 3 weeks only.

Test procedure

1. Transfer 1 ml of a 48 hour MR-VP broth culture to a separate test tube prior to the MR test.

2. Add 1 ml of test reagent to the 1 ml of culture. Aerate by shaking the tube thoroughly.

3. Tests are left at room temperature and final readings are made after 4 hours. Occasional shaking to aerate will speed up a positive reaction. A positive VP test is indicated by the development of a delicate eosin-pink color.

MISCELLANEOUS BIOCHEMICAL TESTS

A. Cetrimide agar.

1. Cetrimide (hexadecyltrimethyl ammonium bromine - Eastman Organic) - prepare a 22.5% solution in distilled water. Store at 4°C.

2. Medium.

Heart infusion agar	40 gm
Cetrimide, 22.5%	4 ml
Distilled water	1000 ml

Autoclave at 121°C for 15 minutes, tube aseptically and slant.

3. Inoculate slants with one drop of a 24 hour infusion broth culture. Incubate at 35-37°C and observe daily for growth up to 7 days.

B. Gelatin.

1. 12% Bacto gelatin in heart infusion broth at pH 7.4. Autoclave at 121°C for 15 minutes.

2. Inoculate with 4 drops of a 24 hour infusion broth culture. Incubate at 35-37°C.

3. Check for liquefaction daily by placing tube in the refrigerator along with an uninoculated control. Tests are read for liquefaction as soon as the control hardens. Tests are held up to 14 days before being reported as negative.

C. Litmus milk.

1. Prepare according to commercial directions.

2. Inoculate with 4 drops of a 24 hour broth culture. Incubate at 35-37°C.

3. Observe daily for 7 days for alkaline reaction (litmus turns blue), acid reaction (litmus turns pink), indicator reduction, acid clot, rennet clot and peptonization.

D. Catalase test.

1. Add several drops (or up to 2-3 ml) of fresh 3% H_2O_2 to a 24-48 hour infusion agar slant or infusion broth culture of the organism to be tested. (Media containing any body fluids will produce false positive reactions.) It is well to have a culture of a known catalase producer as a positive control and a tube of uninoculated broth or agar as a negative control.

2. Observe for active liberation (bubbling or foaming) of O_2 from the H_2O_2 indicating the presence of catalase.

WARNING:

Do not use 30% H_2O_2. It may foam out of the tube, thereby creating a serious contamination hazard.

E. Urea hydrolysis.

1. Christensen's urea agar. Inoculate heavily from a slant culture.

2. Incubate at 35-37°C and observe for development of a pink color denoting urease production. Tests are held up to 7 days.

3. For Brucella hold the inoculated slants at room temperature for 2 hours and then incubate at 35-37°C in 2-10% of CO_2. Observe for development of a pink color at 5 minutes, 2 hours, and after overnight incubation.

F. H_2S production.

1. Inoculate a tube of heart infusion agar or triple sugar iron agar with the culture to be tested.

2. Suspend a strip of lead acetate paper (available commercially) in the tube so that it extends about one inch below the cotton plug; close to, but not touching the medium.

3. Incubate at 35-37°C for 7 days.

4. Examine daily for the degree of blackening of the filter paper strips. Record from trace (slight black) to 4 + (entire strip black).

BILE ESCULIN MEDIUM

Media

1. Medium (available commercially)

Nutrient agar	23.0 gm
Ferric citrate	0.5 gm
Oxgall	40.0 gm
Esculin	1.0 gm
Distilled water	1000.0 ml

2. Autoclave medium at 121°C for 15 minutes. Adjust pH to 6.6. Dispense 6 ml in 16 x 125 SC tubes and slant.

Test procedure

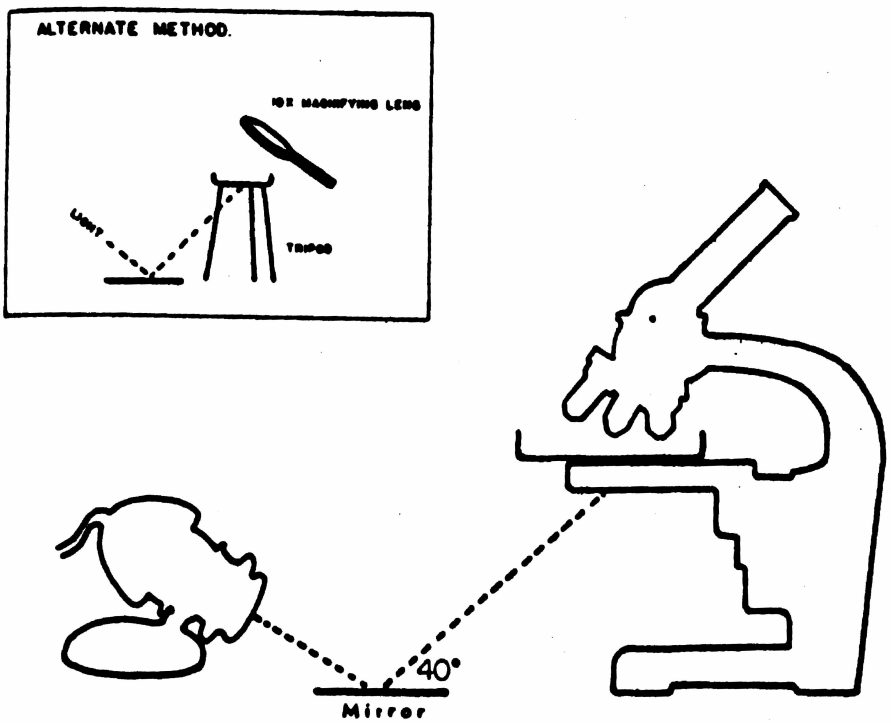
1. Inoculate the test culture lightly and confluently over the surface of the slant. Incubate at 35-37°C up to 48 hours.

2. Groups D and Q streptococci grow on the slant and turn the medium jet black. With the exception of a small percentage of non-groupable alpha hemolytic strains, other streptococci either fail to grow or do not alter the appearance of the medium.

Method of obtaining

OBLIQUE LIGHT

for examining colonies on clear agar media



Adapted from Bearns & Girard, Am. J. Med. Tech., 1959, 121.

GRAM STAIN

SOLUTIONS

	NCDC Modification	Hucker Modification
Crystal Violet		
A. Crystal violet powder (99% dye content)*	13.87 gm	20 gm (90% dye content)
Dissolve in 95% ethanol	200 ml	200 ml
B. Ammonium oxalate	8 gm	8 gm
Distilled water	800 ml	800 ml
Mix A and B. Let sit overnight or for several days until dye goes into solution. Filter through coarse filter paper.		
Gram's Iodine (Lugol's)		
Iodine crystals	1 gm	1 gm
Potassium Iodide	2 gm	2 gm
Distilled water	300 ml	300 ml
Decolorizer		
95% Ethanol		95% Ethanol
Safranin		
Safranin-O	3.41 gm	2.5 gm
95% Ethanol	100 ml	100 ml
Distilled water	900 ml	900 ml

PROCEDURE

1. Make thin smear on glass slide.
2. Air dry and fix GENTLY with heat.
3. Add crystal violet for 1 minute.
4. Rinse with tap water.
5. Add Gram's iodine for 1 minute.
6. Wash slide with tap water.
7. Decolorize with 95% ehtanol for about 30 seconds or until the wash is clear.
8. Counter stain with safranin for 30-60 seconds.

* Dye content will vary. The actual dye content of each lot is given on the dye bottle label. The weight of dye to be used should be adjusted accordingly.

LOEFFLER'S METHYLENE BLUE STAIN

Solution A

Methylene blue (90% dye content)	0.3 gm
Ethyl alcohol, 95%	30.0 ml

Solution B

Dilute potassium hydroxide, 0.01%	100 ml

Mix solutions A and B.

Staining procedure

1. Fix air dried smears by passing through a Bunsen burner flame.
2. Flood smears with stain for 1-2 minutes.
3. Wash lightly, dry, and examine.

LEIFSON'S FLAGELLAR STAIN

SOLUTION A:

Basic fuchsin (certified for flagellar staining)	0.6 g
Ethyl alcohol, 95%	50.0 ml

Shake and let stand overnight to dissolve.

SOLUTION B:

Distilled water	100.0 ml
Sodium chloride	0.75 g
Tannic acid	1.5 g

Combine solutions A and B and mix thoroughly. The stain is ready for use immediately and should remain satisfactory for about 1-2 months when stored at 4-5°C. A precipitate develops on storage which should not be disturbed when the stain is used.

STAINING PROCEDURE:

1. Smears for flagella staining may be made from growth on plates by making a light suspension in distilled water, taking care that only bacterial growth and none of the agar is carried into the suspension. More satisfactory preparations may be obtained with a formalinized, washed cell suspension of the organism from an overnight culture in a non-dextrose containing broth. To prepare the cell suspension, add 0.25 ml formalin to 5 ml of an overnight broth culture, mix, and allow to stand for 15 minutes. Add an equal portion of distilled water to the tubes, mix and centrifuge. Decant the supernatant carefully and wipe off lip of tube. Add distilled water, mix and recentrifuge. Decant as before, resuspend the organisms in 1-2 ml distilled water, and then dilute to a barely turbid suspension.

2. It is essential that clean and grease-free slides be used for flagella staining. Many workers use concentrated sulfuric acid saturated with potassium dichromate to clean the slides; however, new and unopened slides that have been precleaned at time of manufacture will give satisfactory results. The slides, immediately prior to use, must be polished with a soft cotton cloth and then well flamed in the blue flame of a Bunsen burner with the slide to be used next to the flame. A smoky or yellow flame ruins the slides. Draw a line with a wax pencil from the top to the bottom of each slide near the frosted end. The slides should have been flamed uniformly and sufficiently to slightly melt this wax line.

3. To prepare a smear for staining, place prepared slide with the frosted end on an applicator stick to tilt slightly. Place a 3 mm loopful of suspension near the wax line and allow the drop to run to the other end of the slide. The drop will run quite freely if the slide is satisfactory and has been well cleaned. Allow to air dry.

4. Place the slide on a staining rack and add 1 ml of stain warmed to room temperature. Staining is not complete until a metallic sheen has formed on the surface of the stain. This may vary from 6-12 minutes depending on the age of the stain. It is advisable to stain a control slide to determine the staining time and prepare fresh stain if the staining time exceeds 12 minutes.

5. Wash the stained slide with tap water without tilting the slide to drain the stain off. Air dry and examine.

6. If a counterstain is desired, use a 1:10 dilution of Loeffler's methylene blue after washing off the flagellar stain.

RAPID FREEZING AND STORAGE OF BACTERIAL ORGANISMS

Stock cultures ranging from the eugonic enteric organisms to the more fastidious organisms such as Vibrio fetus, Haemophilus species, and Bordetella pertussis can be preserved by quick freezing at -50°C and storage at -45°C. Rate of survival is excellent, and recultivation can be made with relative ease.

The freezing tubes are made of Pyrex glass, size 6 x 50 mm, Corning #9820. It is essential that this particular Pyrex tube be used because of the sudden change in temperature to which it will be subjected. New tubes are boiled in three changes of distilled water to remove any possible deleterious substance. These tubes are then shaken thoroughly to remove the water, dried, plugged, and sterilized in the hot air oven. The cultures can be identified by using 1/4" x 1" strips of waterproof adhesive tape on which the name or number of the culture has been typed or printed, using India ink and the tape affixed 3/8" from the top of the tube.

Storage boxes are constructed of 3/8" marine waterproof plywood. Inside dimensions are 15" long, 6" wide, and 3" deep. In the bottom of the box is a 1" thick piece of 6" x 15" block of styrofoam which has been pre-punched with holes 3/8" on center eacy way. These holes should be slightly less in diameter than that of the tubes to allow a snug fit. Using a 20 penny nail as a punching device has been found to be satisfactory. Before a new box is used, it is placed in the deep freeze and pre-chilled.

Cultures to be frozen are transferred the day before to a 5 ml infusion agar slant in a 15 x 125 mm tube and incubated 18-24 hours to assure the highest number of viable progeny. Any infusion agar that will support the growth of all of the eugonic or non-fastidious organisms such as the enterics, Alcaligenes, Pseudomonas, Bacillus, Listeria, etc., is satisfactory. To grow Haemophilus, add 2 drops of sterile defibrinated rabbit blood to the surface of the slant. For Bordetella pertussis use Bordet-Gengou agar slants. After growth, add 7 drops of sterile defibrinated rabbit blood aseptically to each slant. Capillary pipettes are used to suspend the growth in the blood, then aspirated and delivered to the previously numbered 6 x 50 mm Pyrex freezing tubes. This volume of blood (7 drops) will fill the tubes to approximately 2/3 of their volume. The excess cotton is cut off and the lip flamed.

A small pan is filled with 95% ethyl alcohol to a level proportionate to the top of the suspension within the tubes. Dry ice is now gradually added to the alcohol until the temperature is lowered to -50°C. All of the remaining non-volatilized dry ice is now removed, and the rack containing the tubes is immersed in the alcohol. Quick freezing of the suspensions will take place rather rapidly. It is, however, advisable to leave the tubes in this solution for a minimum of 2-3 minutes. The rack of tubes is then removed from the alcohol, drained momentarily, and placed on a

pre-chilled towel. The actual transference of the tubes to their respective numerical sequence in the storage box should be carried out hurriedly to prevent any possible thawing of the suspensions.

To recultivate the frozen organism, the operator should hold the frozen tube at the meniscus of the blood using the thumb and forefinger and gently rotating the tube until only a small portion has thawed. This may then be removed with a loop or preferably a capillary pipette and inoculated to the most optimum media for growth of the organism. The frozen tube should then be returned immediately to the storage box in the freezer. This procedure should be carried out expeditiously to prevent the entire culture from thawing. If the deep freeze is any considerable distance from the laboratory table, then the culture should be transported in a container with dry ice packing.

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